



## Molecular and bioacoustic differentiation of *Boophis occidentalis* with description of a new treefrog from north-western Madagascar

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### Abstract

We describe a new species of the *Boophis albilabris* group (Anura: Mantellidae) from north-western Madagascar. *Boophis tsilomaro* **sp. nov.** is most similar to *B. occidentalis* from the Isalo Massif, but differs by substantial genetic differentiation, larger size, absence of a turquoise iris colour, presence of a more distinct white stripe along upper lip in life, and longer note duration and lower pulse repetition rate in advertisement calls. Due to its small known range and continuing decline in the extent and quality of its habitat we propose to classify this new species as "Critically Endangered" according to the IUCN criteria. We also describe the advertisement calls of *B. occidentalis* for the first time.

**Key words:** Amphibia, Mantellidae, *Boophis tsilomaro* **sp. nov.**, *Boophis occidentalis*, Madagascar, Isalo, Tsingy de Bemaraha, Sahamalaza Peninsula

### Introduction

The genus *Boophis* comprises 70 described and a considerable number of undescribed species of treefrogs from Madagascar (Cadle 2003; Vieites *et al.* 2009; Glaw *et al.* 2010). *Boophis* belongs to the family Mantellidae, a radiation endemic to Madagascar and the Comoro island of Mayotte. The genus is monophyletic and composed of two main clades that correspond to primarily stream-breeding and pond-breeding species (Glaw & Vences 2006, 2007), and the stream breeders are further divided into eight phenetic species groups.

One clearly monophyletic species group is the *Boophis albilabris* group, with currently three described species: *Boophis albilabris*, *B. occidentalis*, and *B. praedictus* (Glaw *et al.* 2010). These are moderately sized to large treefrogs of mostly green (but sometimes brown) dorsal colour. One of their main characteristics is that after preservation in alcohol, the dorsal colour of many specimens turns purple. In addition, males are characterized by a strong prepollex with a sharp terminal (prepollical) spine, and in breeding condition by distinct keratinized warts or spicules on both the dorsal and ventral surfaces of the body (Andreone 1993; Cadle 1995; Andreone *et al.* 2002). Furthermore, different from most other *Boophis*, males appear to be of same body size or larger than females, suggesting male-male combats during the breeding period which indeed have been observed in one population from Berara forest in north-western Madagascar, assigned to *B. occidentalis* (Andreone *et al.* 2002).

*Boophis occidentalis* was originally described (Glaw & Vences 1994) on the basis of two specimens (probably both subadult females) from Isalo, a sandstone massif in south-western Madagascar with humid relict forests along streams and canyons (Mercurio *et al.* 2008). The species was originally considered to be a subspecies of *Boophis albilabris* (as *B. albilabris occidentalis*) from which it differs by a distinctly smaller body size, presence of dorsolateral yellow stripes, turquoise iris and blue outer iris area. Later, Andreone *et al.*

(2002) reported on two additional populations assigned to this taxon: One of these populations was recorded from the western limestone massif Tsingy de Bemaraha on the basis of one male specimen previously collected by R. Jesu and L. Emanuelli (Emanuelli & Jesu 1995) that already had been discussed by Andreone (1993). This specimen was characterized, on the basis of a life photograph supplied to FA, by the typical turquoise iris, yellow dorsolateral stripes, and a dense coverage of keratinized spicules on the dorsal surface. The second population was recorded from the north-western transitional Berara forest (on Sahamalaza Peninsula). Here a large number of individuals were observed of which none had turquoise colour in the iris. Breeding males had as well the dorsum covered with small and thick black spicules. Because (1) the Berara specimens had an advertisement call different from that of *Boophis albilabris*, and (2) these specimens were considered to represent *B. albilabris occidentalis* despite their differences in iris colouration, Andreone *et al.* (2002), elevated this taxon to full species status as *B. occidentalis*.

In the last six years, we have been able to collect further material and data on these frogs that shed new light on their systematics: (1) One specimen provided by E. Edwards in 2001 had the same morphology of typical *B. occidentalis* from Isalo but was said to have been collected from the “Antoetra region” in the Malagasy highlands (without detailed collecting data). Although this locality is dubious and needs confirmation, it suggests a possibly wider distribution of this species. (2) In 2006, we obtained two tadpoles of *B. occidentalis* from the Tsingy de Bemaraha Massif, although unfortunately no further adults from this population were observed. (3) During intensive fieldwork of F. Andreone and colleagues at Isalo (e.g., Andreone *et al.* 2007; Mercurio *et al.* 2008), numerous additional natural history data of *B. occidentalis* from its type locality became available; eventually M. Vences and J. Glos, in 2007, succeeded in recording the advertisement calls of this species in Isalo.

The new data further corroborated the constancy of the chromatic differences between the Isalo and Berara populations, and also indicated differences in advertisement calls. Here we analyse the bioacoustic divergence between the Isalo and Berara populations, provide molecular data for specimens from all known *B. occidentalis* populations, and conclude that the Berara population represents a genetically, bioacoustically, and morphologically differentiated new species.

## Materials and Methods

Frog specimens were collected at night by opportunistic searching and by locating calling males, using torches and head lamps. Specimens were euthanized in a chlorobutanol solution, fixed in 5% formalin or 95% ethanol, and preserved in 70% ethanol. Specimens are deposited in the collections of the Museo Regionale di Scienze Naturali, Torino, Italy (MRSN), Museo Civico di Storia Naturale, Genova, Italy (MSNG), Université d'Antananarivo, Département de Biologie Animale, Antananarivo, Madagascar (UADBA), Zoologisches Forschungsmuseum Alexander Koenig, Bonn, Germany (ZFMK), Zoölogisch Museum Amsterdam, The Netherlands (ZMA), and the Zoologische Staatssammlung München, Germany (ZSM). FGMV, FGZC and ZCMV refer to F. Glaw and M. Vences field numbers; FAZC refers to F. Andreone field numbers; LR refers to L. Raharivololoniaina field numbers.

Morphological measurements (in millimetres) were all done by M. Vences with digital callipers (precision 0.01 mm) to the nearest 0.1 mm. Abbreviations are: SVL (snout-vent length), HW (maximum head width), HL (head length), ED (horizontal eye diameter), END (eye-nostril distance), NSD (nostril-snout tip distance), NND (nostril-nostril distance), TD (horizontal tympanum diameter), TL (tibia length, actually referring not to the tibia bone but to the shank), HAL (hand length), HIL (hindlimb length), FOL (foot length), FOTL (foot length including tarsus), FORL (forelimb length), and RHL (relative hindlimb length). The definition of these measurements, terminology and description scheme follow Glaw *et al.* (2001), and Glaw & Vences (1997) for eye colouration. Webbing formulae follow Blommers-Schlösser (1979).

Calls of *B. occidentalis* were recorded using an Edirol R09 digital recorder. Call recordings were analyzed on a personal computer with Windows XP Professional operating system using the software Cool Edit Pro 2.0 and Raven Pro 1.3. Spectrograms were obtained in Raven at Hanning window function with 512 bands resolution. Temporal measurements are given as range, with mean  $\pm$  standard deviation in parentheses.

Tissue samples of all species of the *Boophis albilabris* group were taken in the field and preserved in 95–99% ethanol. DNA was extracted and a fragment of the mitochondrial 16S rRNA gene amplified using primers 16Sar-L and 16Sbr-H of Palumbi *et al.* (1991), following standard protocols (see Vences *et al.* 2005; Glaw *et al.* 2010), and subsequently resolved on automated sequencers. Sequences were aligned by eye, and their alignment required the assumption of only a single indel. Phylogenetic analyses were performed using the programs PAUP 4b10\* (Swofford 2002), and Mrbayes 3.1 (Ronquist & Huelsenbeck 2003). We performed Bayesian, maximum likelihood (ML), and maximum parsimony (MP) analyses in order to check for consistency in the results from different methods that are based on different assumptions of molecular evolution. MP analyses were performed in PAUP\* 4b10 using heuristic searches with tree-bisection-reconnection (TBR) branch swapping, step addition starting tree, and random addition sequence with 10 replicates. ML analyses were carried out in PAUP\* after determining the appropriate substitution model (a general time-reversible GTR+I+G model) using hierarchical likelihood ratio tests in Modeltest (Posada & Crandall 1998). Under both MP and ML we ran a bootstrap test with 2000 replicates (MP) and 500 replicates (ML). For the Bayesian analysis, we used MrModeltest version 2.2 (Nylander 2004) to choose the appropriate model of sequence evolution for each partition (again, a GTR+I+G model was selected). We used a neighbour-joining (NJ) tree with Jukes–Cantor 69 substitution model of the whole data set as starting tree. Analyses consisted of four Markov chains that ran for 10 million generations, sampled every 100 generations, with a random starting tree and default priors. The burn-in was empirically estimated by plotting  $-\ln L$  against the generation number, and the trees corresponding to the first 5 million generations were conservatively discarded.

## Results

### Molecular phylogeny and divergences

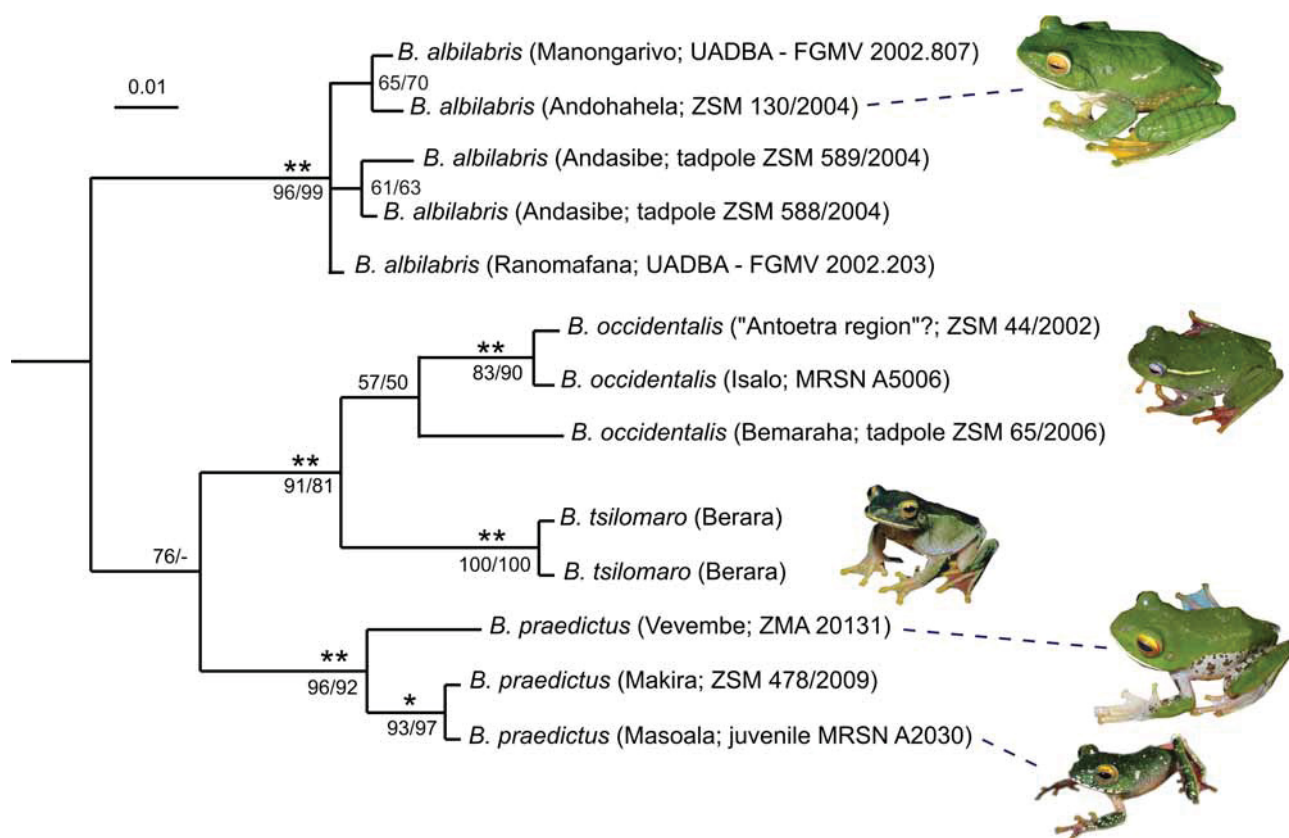
The different tree reconstruction methods yielded largely congruent results. Of a total of 494 aligned nucleotides, 424 characters were constant and 40 were parsimony-informative. The tree (figure 1) places all four populations that in the past have been assigned to *Boophis occidentalis* (Isalo, “Antoetra region”, Bemaraha, and Berara) as a monophyletic group with high support and provides weak support for a clade containing the specimens from Isalo, “Antoetra region”, and Bemaraha. The genetic divergences between these clades were substantial: 2.5–2.7% pairwise uncorrected p-distance between Bemaraha and Isalo/“Antoetra region”, 3.8% between Bemaraha and Berara, and 3.1–3.3% between Isalo/“Antoetra region” and Berara.

### Morphological differences between populations

The molecular differentiation of populations assigned to *B. occidentalis* correlates with some, subtle, morphological and chromatic differences. The most distinct difference, as already reported by Andreone *et al.* (2002) is the lack of turquoise iris colour in the population of Berara. Furthermore, the fact that so far in Isalo no specimens with a dense coverage of black dorsal spicules have been observed and that even calling males had few spicules, indicates that these spicules may be more weakly expressed in this locality. Summarizing the most obvious differences (see also tables 1–2), the populations can be characterized as follows (not considering the single specimen from the dubious provenance “Antoetra region”): (1) Specimens from Isalo have only green dorsal colour with turquoise iris periphery and blue iris colouration, a rather indistinct white stripe along upper lip, and breeding males as far as known have a weak expression of poorly keratinized dorsal spicules (see figure 2a), although it is uncertain whether highly motivated males in an explosive breeding situation may show such dermal structures. (2) Specimens from Berara have green or brown dorsal colour with a light brown iris and blue iris periphery, a distinct white stripe along upper lip, and breeding males have a dense dorsal coverage of black spicules (figure 2b). (3) Frogs from Tsingy de Bemaraha are as

**TABLE 1.** Morphometric measurements (in mm) of holotype and seven paratype specimens of *Boophis tsilomaro* from its type locality (Berara forest), and comparative specimens of *B. occidentalis* from its type locality (Isalo National Park; ZSM 539/1999, ZSM 2314-2315/2007 and 2402/2007), and from the unconfirmed locality “Antoetra region” (ZSM 44/2002). For abbreviations of measurements see Materials and Methods. Relative hindlimb length (RHL) is given as the point reached by the tibiotarsal articulation when the hindlimb is adpressed along the body: (1) posterior eye corner, (2) eye centre, (3) anterior eye corner, (4) nostril. M, male; F, female; SA, subadult; HT, holotype; PT, paratype.

Voucher	Status	Sex	SVL	HW	HL	ED	TD	ENS	NSD	NND	HAL	FORL	HIL	FOTL	FOL	TL	RHL
<i>B. tsilomaro</i> sp. nov.																	
MRSN A2002	HT	M	55.6	19.0	19.2	5.9	4.0	4.8	3.8	5.9	17.0	33.6	87.3	41.9	24.4	27.8	2
MRSN A1997	PT	M	56.5	19.2	18.5	6.0	3.8	4.3	3.4	5.9	17.1	32.8	87.4	38.9	23.3	25.6	1
MRSN A1998	PT	M	53.4	17.9	16.9	5.7	3.9	4.1	4.0	5.5	14.7	31.4	79.2	35.8	21.8	25.2	2
MRSN A1999	PT	M	64.1	20.7	20.4	5.9	4.3	4.8	4.5	6.6	18.5	37.7	98.9	44.1	25.8	29.5	2
MRSN A2001	PT	M	61.3	20.8	20.2	6.2	4.7	4.6	4.1	6.8	17.2	36.1	98.9	44.4	26.0	28.4	2
MRSN A2003	PT	M	52.8	16.7	17.1	5.7	4.0	4.2	3.9	5.7	14.2	28.9	77.8	34.6	20.8	24.1	2
MRSN A1996	PT	F	49.1	17.9	17.6	5.4	3.8	4.2	3.8	5.4	14.3	29.8	79.3	33.6	19.5	24.8	3
MRSN A2004	PT	F	57.9	20.3	20.0	5.5	4.3	5.0	4.2	6.0	17.6	36.4	92.6	41.7	25.0	27.8	3
<i>B. occidentalis</i>																	
ZSM 2314/2007	---	M	46.0	16.6	15.6	5.0	3.7	3.4	4.5	5.1	14.3	27.3	70.6	31.3	19.3	21.5	3
ZSM 2402/2007	---	M	52.4	18.4	17.0	5.5	4.1	3.4	4.1	5.2	16.6	31.1	77.7	34.6	21.4	23.2	2
ZSM 539/1999	PT	F (SA)	38.4	14.3	13.8	4.8	2.9	3.6	3.4	3.8	12.3	23.9	63.4	26.8	15.8	19.1	4
ZSM 44/2002	---	F (SA)	36.4	12.3	12.1	4.6	2.2	2.5	3.2	4.8	11.2	22.3	57.1	24.7	15.0	16.8	2
ZSM 2315/2007	---	F	48.3	15.8	15.7	5.3	3.9	3.3	3.4	5.0	13.8	29.0	75.1	32.3	20.1	22.8	3



**FIGURE 1.** Bayesian inference phylogenetic tree of species in the *Boophis albilabris* group based on 494 nucleotides of the mitochondrial 16S rRNA gene. Asterisks denote Bayesian posterior probabilities of 0.95 and higher (one asterisk) and 0.99 and higher (two asterisks). Numbers at nodes are bootstrap values in percent of ML and MP analyses, respectively (500 and 2000 replicates). *Boophis goudoti*, a member of the *B. goudoti* group, was used as the outgroup (not shown). The inset pictures show representative photos of the respective species (from top to bottom): *B. albilabris*, male ZSM 130/2004; *B. occidentalis*, male ZSM 2314/2007; *B. tsilomaro*, male from Berara (not collected), *B. praedictus* (female ZSM 478/2009; *B. praedictus*, probable juvenile MRSN A2030). Size differences between inset photos are indicative of original sizes of the frogs but are not to scale.

far as known green in life with a turquoise iris and blue iris periphery, a distinct white stripe along upper lip, and with a dense coverage of black spicules in breeding males (based on the photograph of one specimen, supplied by R. Jesu).

To provide additional data on the enigmatic Tsingy de Bemaraha population, we were able to examine one preserved male specimen (MSNG 48586) collected by L. Emanuelli and R. Jesu but apparently not identical to the photographed specimen. Its SVL is around 44 mm, and the overall colouration, after more than 15 years from its collecting, is purple on the back and dirty whitish on the belly. Two thin light lines run from the nostrils to the upper parts of the eyelids and end after around 16 mm on a very slightly elevated dorso-lateral ridge, continuing with a few lighter warts on the back. Three irregularly placed white spots are present on the lower back. The back (between the dorsolateral ridges) is covered by rather distinct dark spicules, which are also present on the lower jaws and on the breast. Similar spicule-covered surfaces are also visible on the upper part of the prepollex, on the first and on the second finger. The prepollex is with terminal spine. Eye colouration appears dark (pupils being greyish, and outer iris is blackish), but it is impossible to hypothesize on the actual iris colouration in life of this specimen. The overall colouration and general skin morphology appear similar to those observed in the specimens from Berara.



## Vocalizations of *Boophis occidentalis* and bioacoustic comparisons

Advertisement calls of *B. occidentalis* were recorded from a single, isolated male (ZSM 2314/2007 [ZCMV 5551]) in the Namazaha Valley at Isalo National Park on 15 February 2007, ca 21:00 h, at an estimated air temperature of 25° C. When the recordings were taken, no additional males were calling or interacting with the calling male at this site. Nevertheless, the male appeared to be highly motivated and was repeating its call multiple times. Furthermore, at other sites along the same stream, at least 100 m from the first site, we heard additional single males (at least one specimen but probably several) emitting the same calls, but these specimens were too difficult to approach and collect due to the depth and current of the stream.

The calls (figure 3) were unharmonious, pulsed notes emitted in short regular series. Notes were composed of 50–66 ( $57 \pm 4.3$ ,  $n=12$ ) pulses. Note duration was 856–1112 ms ( $996 \pm 68.8$  ms,  $n=12$ ), duration of intervals between notes of a regular note series was 534–1164 ms ( $785 \pm 236.8$  ms,  $n=10$ ), with longer intervals between regular note series (2073 and 4210 ms,  $n=2$ ). Duration of pulses was 7–14 ms ( $10.8 \pm 2.1$  ms,  $n=14$ ), duration of intervals between them was 2–13 ms ( $5.1 \pm 3.0$  ms,  $n=14$ ). No click pulses were recognized. Pulse repetition rate was 53–60 pulses per second. Frequency was 500–1050 Hz, with a dominant frequency band between 560–750 Hz.

A comparison with calls from Berara which have been described (under the name *B. occidentalis*) and recorded in a chorus situation (Andreone *et al.* 2002) reveals that the latter differs from the Isalo calls by less pulses per note (26–34 vs. 50–66), shorter note duration (262–362 ms vs. 856–1112 ms), a higher pulse repetition rate (90–109 vs. 53–60 per second), and higher frequency range (900–2000 Hz vs. 500–1050 Hz).

Because the data from Isalo are based on vocalizations of a single specimen, the variation of calls of this population still needs to be fully assessed. However, (1) at least one additional male was heard at Isalo and the calls sounded very similar to that of the recorded specimen (in fact, it is possible for human receivers to easily recognize the temporal call differences between the populations), and (2) of numerous males recorded at Berara, none emitted the slow and long calls recorded at Isalo. We therefore conclude that these differences very probably represent a constant bioacoustic differentiation among these populations, confirming the specific distinctness of the candidate species from Berara which is described in the following:

### *Boophis tsilomaro* sp. nov.

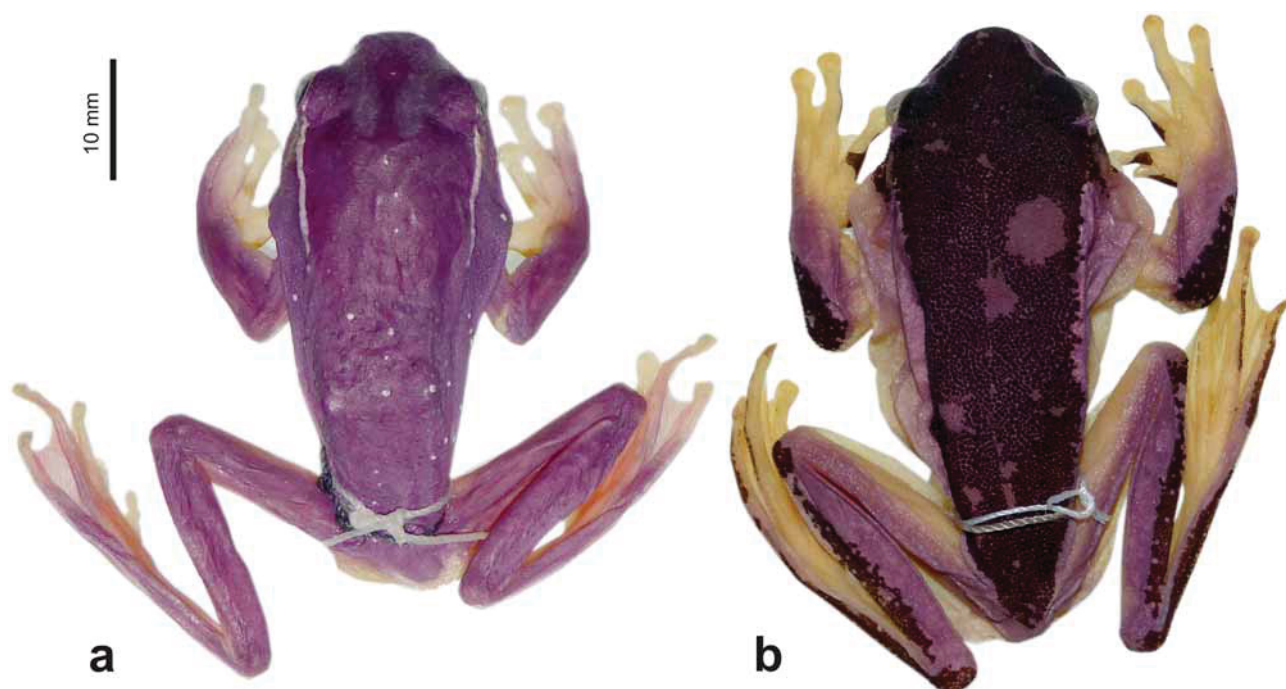
(figures 2 and 4)

**Etymology.** The specific name is used as a noun in apposition and is composed of the Malagasy words "tsilo", meaning "spine", and "maro", meaning "many". The name makes reference to the typical keratinized spicules on the dorsum and chest of breeding males.

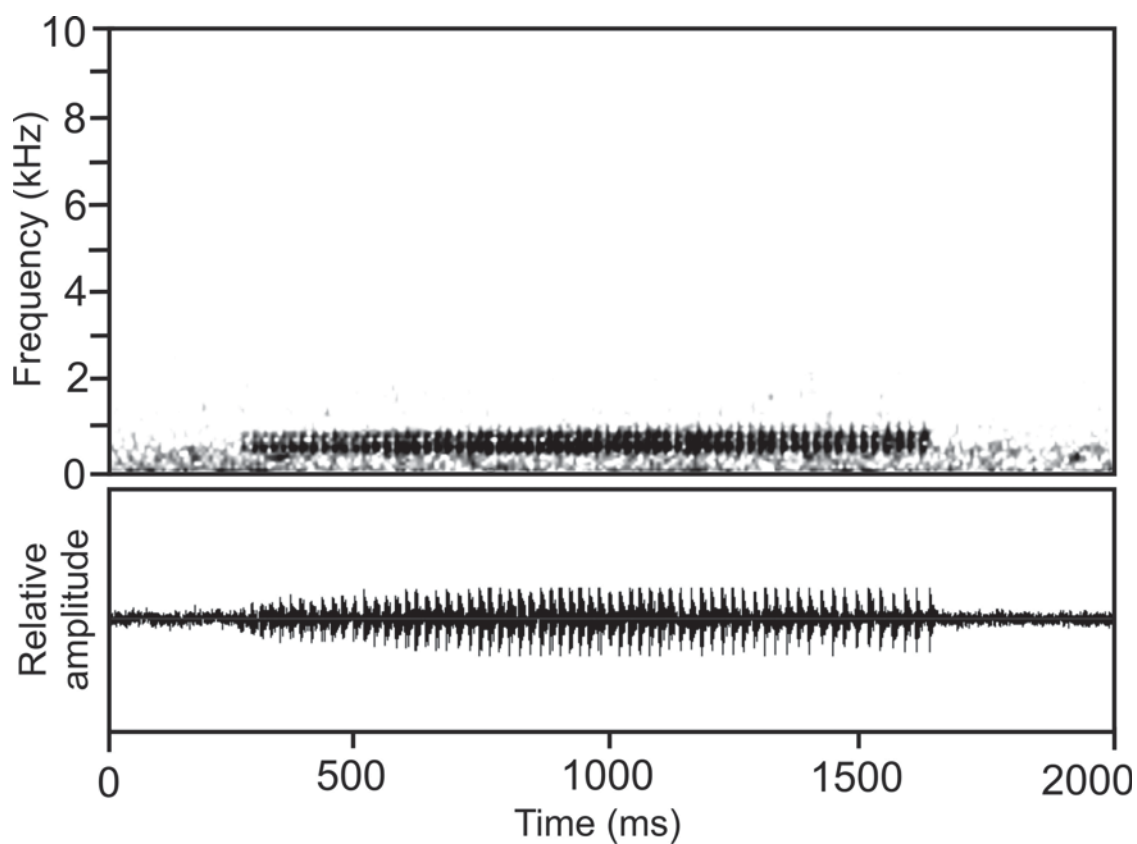
**Remark.** This species has been referred to as *Boophis occidentalis* by Andreone *et al.* (2002) and Vences *et al.* (2006), as *Boophis* sp. aff. *occidentalis* by Glaw & Vences (2007:166–167) and as *Boophis* sp. 4 in Vieites *et al.* (2009).

**Holotype.** MRSN A 2002 (FAZC 10667, formalin fixed), adult male collected at Berara Forest (Sahamalaza Peninsula, north-western Madagascar), 14°8'18.55"S and 47°8'54.92"E, 170 m a.s.l., Mahajanga Province, Analalava Fivondronana, Amboloboza Firaiana and western part of the Befotaka Firaiana, on 20 February 2000 by F. Andreone, J. E. Randrianirina & M. Vences.

**Paratypes.** MRSN A1996 (FAZC 10581), adult female; MRSN A2001 (FAZC 10667, formalin fixed), adult male; MRSN A2004 (FAZC 10666, formalin fixed), adult female; MRSN A1997 (FAZC 10653), MRSN A1998 (FAZC 10659), MRSN A 1999 (FAZC 10661), MRSN A2003 (FAZC 10695), four adult males. All the individuals have been collected at Berara Forest (Sahamalaza Peninsula, north-western Madagascar), 14°8'18.55"S and 47°8'54.92"E, 170 m a.s.l., Mahajanga Province, Analalava Fivondronana, Amboloboza Firaiana and western part of the Befotaka Firaiana, on 20–21 February 2000 by F. Andreone, J. E. Randrianirina & M. Vences.

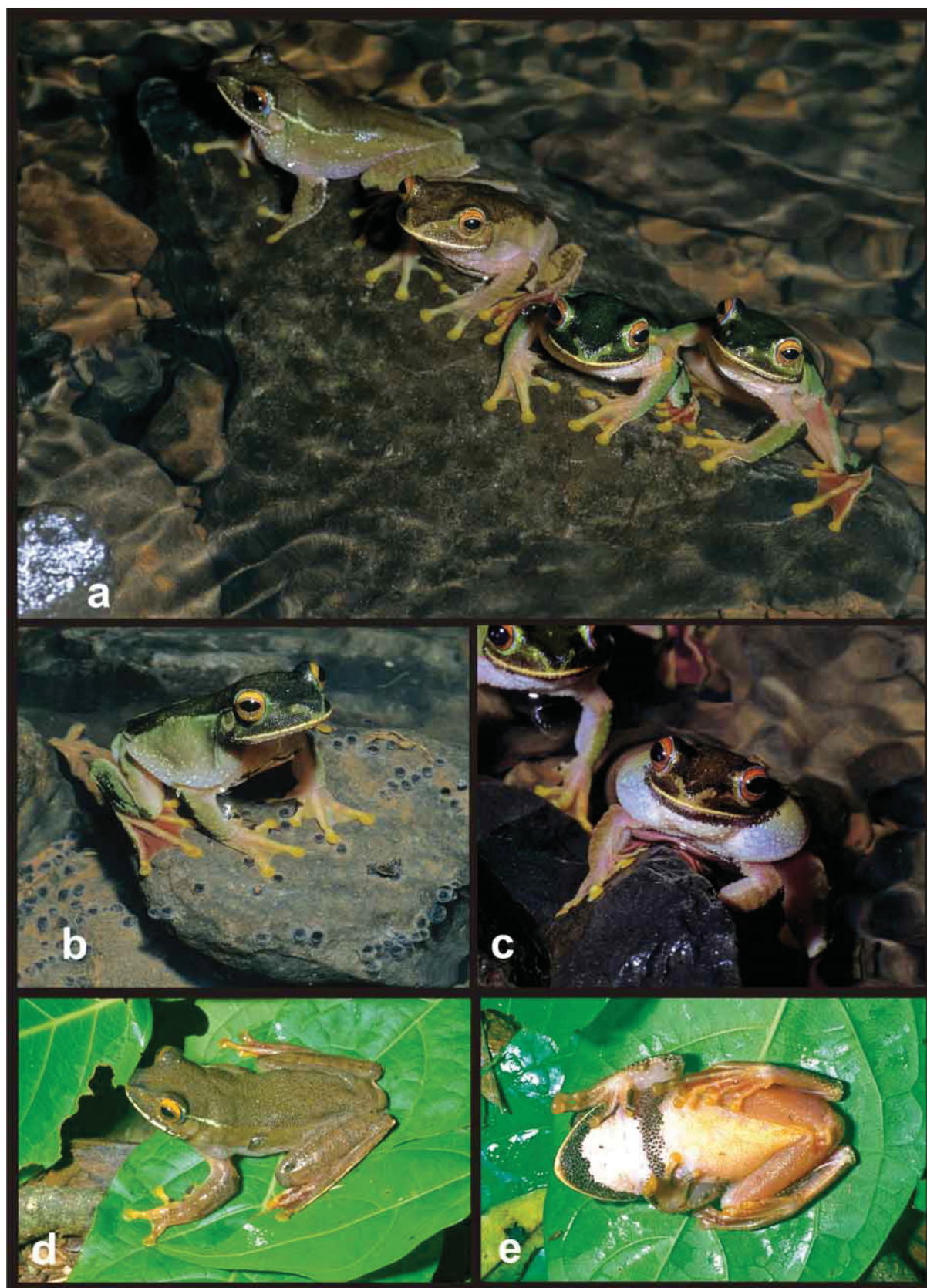


**FIGURE 2.** Preserved adult male specimens of (a) *Boophis occidentalis* (ZSM 2314/2007 from Isalo) and (b) *Boophis tsilomaro* (holotype MRSN A2002 from Berara forest). Both males were collected in breeding condition during emission of advertisement calls; note the slightly larger size of *B. tsilomaro* and the dense coverage of its dorsum with keratinized spicules.



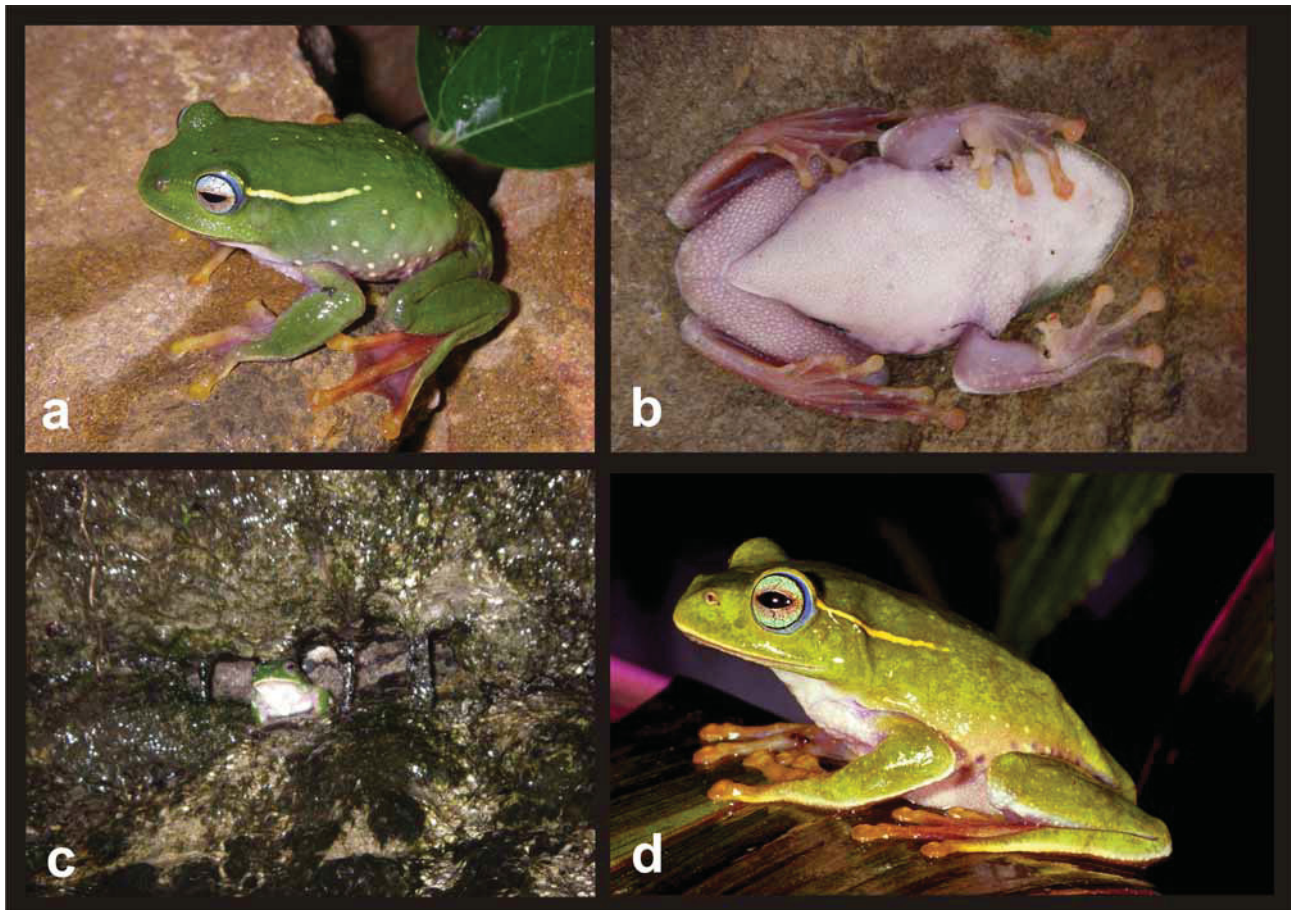
**FIGURE 3.** Sonogram and corresponding oscillogram of one note of a call of *Boophis occidentalis* (ZSM 2314/2007) from Isalo National Park.





**FIGURE 4.** Photographs of *Boophis tsilomaro* in life, photographed at Berara forest in February 2000. (a) Two brownish-coloured and two green-coloured males as part of a breeding aggregation sitting in a shallow and slow-flowing part of a stream; (b) green-coloured male in the stream, visible on the stones are eggs of this species; (c) calling male; (d) brown-coloured male in dorsolateral view; (e) ventral view of same specimen as in (d).





**FIGURE 5.** Photographs of *Boophis occidentalis* from Isalo National Park in life. (a–b) Male specimen ZSM 2314/2007 in dorsolateral and ventral view, and (c) same specimen at its calling position in a small cavity in a rock above a stream, photographed on 15 February 2007; (d) holotype (ZFMK 57383) collected on 29 January 1994.

**Additional material.** Eggs and larval stages (all from Berara Forest): MRSN A2005 (tadpoles, preserved on 26 February 2000, at 11 h); MRSN A2006 (tadpoles, preserved on 4 March 2000, at 15 h); MRSN A2007 (tadpoles, preserved on 7 March 2000, at around 14–16 h); MRSN A2008 (tadpoles, preserved on 9 March 2000, at about 18 h); MRSN A2009 (tadpoles, preserved on 26 March 2000, at about 20–22 h); MRSN A2010 (tadpole, preserved on 9 April 2000); ZSM 57/2001 (embryos, preserved on 22 February 2000, at about 10 h); ZSM 54/2001 (embryos, preserved on 23 February 2000, at about 9.15 h), ZSM 56/2001 (tadpoles, preserved on 24 February 2000, at about 22 h); ZSM 192/2001 (eggs, laid on 20 February 2000, fixed at about 24 h).

**Diagnosis.** Assigned to the genus *Boophis* based on the presence of an intercalary element between ultimate and penultimate phalanges of fingers and toes (verified by external examination), enlarged terminal discs of fingers and toes, lateral metatarsalia separated by webbing, absence of outer metatarsal tubercle, molecular phylogenetic relationships (see Vieites *et al.* 2009 for a complete molecular analysis of *Boophis*), and overall similarity to other *Boophis* species. Assigned to the *Boophis albilabris* group based on the following combination of characters: large size (male SVL 53–64 mm); well developed webbing between fingers; presence of vomerine teeth; green colouration in life and colouration in preservative with a purple shade; presence of a white line along upper lip; molecular phylogenetic relationships (Vieites *et al.* 2009); and overall similarity to *B. albilabris*. *Boophis tsilomaro* differs from the other described species in the *Boophis albilabris* group by substantial genetic differentiation, with pairwise 16S divergences of 3.1–3.8% to *B. occidentalis*, and 4.5–6.0% to other species of the group. It further differs from *B. praedictus* and *B. albilabris* by the presence in most specimens of light dorsolateral lines which run from the eyes to the mid-flanks, by smaller size (adult SVL 53–64 mm vs. 62–89 mm and 76–93 mm, respectively) and by the colouration of the iris periphery (bluish vs. reddish, whitish or greenish). It mostly resembles its sister species *B. occidentalis*.

(see figure 5) from which it differs by a different iris colouration (outer iris ring orange-brown vs. metallic turquoise blue), a less distinct light dorsolateral stripe from the eye to mid-flanks (especially in preservative), larger size (SVL 53–64 mm vs. 46–52 mm SVL), a more distinct white line along the upper lip, and distinct differences in advertisement calls (see above).

**Description of the holotype.** Adult male in breeding condition. Body moderately slender; head slightly longer than wide, slightly wider than body; snout rounded in dorsal view, obtuse in lateral view, nostrils directed laterally, nearer to tip of snout than to eye; canthus rostralis distinct, slightly concave in dorsal view, loreal region slightly concave; tympanum distinct, rounded, TD 67% of ED; supratympanic fold thin, distinct; vomerine odontophores distinct, well separated in two elongated patches, positioned posteromedial to choanae; choanae medium-sized, elongated. Tongue posteriorly bifid, free. Arms slightly thickened, with a white dermal edge from elbow to the lateral base of the finger. Subarticular tubercles single, round; metacarpal tubercles not recognizable; fingers broadly webbed; webbing formula 1(1), 2i(1.5), 2e(0), 3i(2), 3e(0), 4(0); relative length of fingers  $1 < 2 < 4 < 3$  (finger 2 distinctly shorter than finger 4); finger discs enlarged. A bony prepollex at the base of the first finger. Black keratinized nuptial pads on the base of the prepollex, and on the inner sides of fingers 1–3 as far as they are free of web, reaching the base of terminal finger discs. Hindlimbs slender; tibiotarsal articulation reaching centre of eye when hindlimb is pressed along body; lateral metatarsalia separated by webbing; inner metatarsal tubercle small, distinct, elongated; no outer metatarsal tubercle; toes almost fully webbed; webbing formula 1(0), 2i(0.25), 2e(0), 3i(0.5), 3e(0), 4i(0.5), 4e(0.5), 5(0); relative length of toes  $1 < 2 < 5 = 3 < 4$ ; toe discs enlarged. Skin granular on dorsal surfaces, especially where covered by black keratinized skin (almost whole head and back from snout to vent, external parts of lower arm, lower leg, tarsus and lateral sides of toes 4 and 5). Cloaca difficult to recognize, apparently concealed by a skin fold and oriented anteroventrally. Skin largely folded on the flanks, slightly granular on throat and chest, on belly and ventral surfaces of thighs except for a keratinized band along the lower lip and a second band of similar width (3–5 mm). For measurements see table 1.

After almost ten years in preservative, ground colour of dorsal upper surfaces is purple, which however is superimposed by large areas of skin covered by blackish keratinized spicules. A purple band below the canthus rostralis runs from snout tip to eye and is continued on ring surrounding the eye ventrally. The tympanum is purplish-brown. A narrow white band runs along the upper lip. Dorsal sides of fingers and toes pale yellowish. Upper flanks purple, lower flanks yellowish. A continuous pale yellowish narrow stripe on the lateral edges of lower arm and lateral finger and on the heel, and a discontinuous one along tarsus and lateral toe. All ventral surfaces pale yellowish except the areas along the lower lip, on the chest between the arms, and to a much lesser extent on the ventral side of the tarsus which are covered by blackish keratinized spicules. A light dorsolateral stripe from the eye to mid-flanks is not recognizable. Colouration in life unknown, but it might be expected that all purplish parts have been green.

**Variation.** Measurements of the paratypes are given in table 1. Most of the paratypes generally resemble the holotype in morphology and colouration (especially all other males which have similar areas of black keratinized skin), but several differences are evident. MRSN A2001 has a brown colouration without any traces of purple. The dorsal skin is granular, but only fragmentary spicule cover is present on the sides of the head, along lower lip, on chest and elbows and on few other small patches of the dorsal surface. This may indicate that the level of sexual activity was already decreasing when the frog was collected, although black nuptial pads are still present on fingers 1–3. MRSN A2004 and MRSN A1996 are females with rather uniform purple dorsal colouration and distinct light dorsolateral stripes behind the eye. MRSN A1996 has well developed eggs with a light and a dark pole in the body cavity. The body cavity of most paratypes (all except MRSN A1997 and MRSN A2001) was opened for further studies. The life colouration of several specimens is shown in figure 4. According to these photographs dorsal colouration varies from green to olive grey, partly superimposed by blackish keratinized spicules in males. Inner iris ring brown, outer iris ring golden, posterior iris periphery blue. The webbing between the toes is red, the dorsal side of fingers and toes is yellow. Throat whitish, belly and ventral parts of limbs beige to orange-brown.

**Natural history.** All available natural history data were published by Andreone *et al.* (2002): Individual age assessed by skeletochronology ranged from 4 to 11 years. Breeding behaviour was observed at a seasonal

stream in subhumid forest after heavy rainfalls: Five choruses of eight to 90 males aggregated in shallow brook sections. Calling males were engaged in scramble battles. A description of the call is provided above and in Andreone *et al.* (2002). The call is documented in Vences *et al.* (2006) under the name *Boophis occidentalis*. Only two females were found. A pair laid a large number of eggs, attached as a single layer of isolated eggs on submerged stones. Tadpoles reared from these eggs had the typical morphology of stream-breeding *Boophis* species with a 6(2–6)/3 keratodont formula and a relatively depressed body; see Andreone *et al.* (2002) for a detailed tadpole description.

**TABLE 2.** Diagnostic characters and morphometric measurements (in mm) of adult males and females of *Boophis occidentalis* and *B. tsilomaro* **sp. nov.**, summarized as mean (in bold)  $\pm$  standard deviation, and minimum-maximum in parentheses. The population from the Tsingy de Bemaraha is not considered here (see text for a discussion of their characters). For abbreviations, see Materials and Methods and caption to Table 1. Summarized from original data in Table 1. States of the last three characters refer to both males and females of each species.

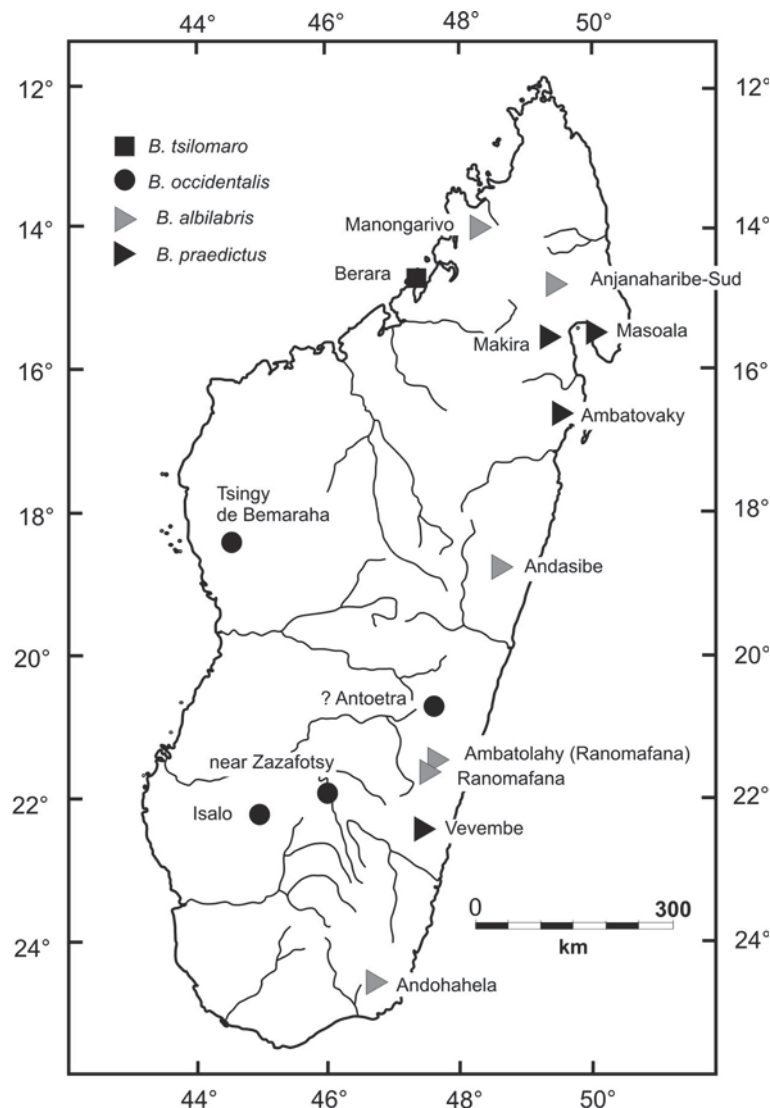
	<i>B. tsilomaro</i>		<i>B. occidentalis</i>	
	males	females	males	females
N	6	2	2	1
SVL	57.3 $\pm$ 4.5 (52.8–64.1)	53.5 $\pm$ 6.2 (49.1–57.9)	49.2 $\pm$ 4.5 (46.0–52.4)	48.3
HW	19.1 $\pm$ 1.6 (16.7–20.8)	19.1 $\pm$ 1.7 (17.9–20.3)	17.5 $\pm$ 1.3 (16.6–18.4)	15.8
HL	18.7 $\pm$ 1.5 (16.9–20.4)	18.8 $\pm$ 1.7 (17.6–20.0)	16.3 $\pm$ 1.0 (15.6–17.0)	15.7
ED	5.9 $\pm$ 0.2 (5.7–6.2)	5.5 $\pm$ 0.1 (5.4–5.5)	5.3 $\pm$ 0.4 (5.0–5.5)	5.3
TD	4.1 $\pm$ 0.3 (3.8–4.7)	4.1 $\pm$ 0.4 (3.8–4.3)	3.9 $\pm$ 0.3 (3.7–4.1)	3.9
ENS	4.5 $\pm$ 0.3 (4.1–4.8)	4.6 $\pm$ 0.6 (4.2–5.0)	3.4 $\pm$ 0.0	3.3
NSD	4.0 $\pm$ 0.4 (3.4–4.5)	4.0 $\pm$ 0.3 (3.8–4.2)	4.3 $\pm$ 0.3 (4.1–4.5)	3.4
NND	6.1 $\pm$ 0.5 (5.5–6.8)	5.7 $\pm$ 0.4 (5.4–6.0)	5.2 $\pm$ 0.1 (5.1–5.2)	5.0
HAL	16.5 $\pm$ 1.7 (14.2–18.5)	16.0 $\pm$ 2.3 (14.3–17.6)	15.5 $\pm$ 1.6 (14.3–16.6)	13.8
FORL	33.4 $\pm$ 3.2 (28.9–37.7)	33.1 $\pm$ 4.7 (29.8–36.4)	29.2 $\pm$ 2.7 (27.3–31.1)	29.0
HIL	88.3 $\pm$ 3.2 (77.8–98.9)	86.0 $\pm$ 9.4 (79.3–92.6)	74.2 $\pm$ 5.0 (70.6–77.7)	75.1
FOTL	40.0 $\pm$ 4.2 (34.6–44.4)	37.7 $\pm$ 5.7 (33.6–41.7)	33.0 $\pm$ 2.3 (31.3–34.6)	32.3
FOL	23.7 $\pm$ 2.1 (20.8–26.0)	22.3 $\pm$ 3.9 (19.5–25.0)	20.4 $\pm$ 1.5 (19.3–21.4)	20.1
TL	26.8 $\pm$ 2.1 (24.1–29.5)	26.3 $\pm$ 2.1 (24.8–27.8)	22.3 $\pm$ 1.2 (21.5–23.2)	22.8
Dorsal spicules	black, densely covering most of dorsum in breeding males	absent	apparently not or only slightly black pigmented, scattered on dorsum of breeding males (with spicules at Bemaraha; see text)	absent
Advertisement calls	26–34 pulses per note, note duration 262–362 ms, 90–109 pulses per second, frequency 900–2000 Hz	---	50–66 pulses per note, note duration 856–1112 ms, 53–60 pulses per second, frequency 500–1050 Hz	---
Dorsolateral line from eye to mid-flanks	usually indistinct, thin, pale yellow		usually distinct, bright yellow	
White line along upper lip	usually distinct		usually indistinct or absent	
Colour of outer iris area	yellowish brown		turquoise	

**Distribution.** Only known from the type locality at Berara (figure 6). The population from Tsingy de Bemaraha, which resembles both *Boophis tsilomaro* and *B. occidentalis*, needs further study but despite its similarity in external morphology is probably not referable to the new species described herein based on the combined evidence from its life colouration, relative small size of 44 mm (compare with table 1) and molecular relationships (see phylogenetic tree in figure 1).



## Discussion

Madagascar's biota is characterized by an extraordinary microendemism, with many species restricted to only very small ranges (Vences *et al.* 2009). The diversification mechanisms that led to the high diversity and local endemism of species are poorly understood, although several hypotheses invoking river barriers, watershed refugia, mountain refugia, and ecogeographic constraints have been formulated (e.g., Raxworthy & Nussbaum 1995; Pastorini *et al.* 2003; Wilmé *et al.* 2006; see summary in Vences *et al.* 2009).



**FIGURE 6.** Map of the distribution of species in the *B. albilabris* group. Note that for *B. albilabris*, many more localities are known and only those are shown from where molecular or bioacoustic data are available (see Andreone *et al.* 2002; Glaw *et al.* 2010). The locality Antoetra for *B. occidentalis* refers to an unverified record from the “Antoetra region” and is in need of confirmation, and the record from Zazafotsy is based on a dead specimen observed by us (from Glaw & Vences 2007).

In amphibians, some groups such as cophyline microhylids in north-eastern and south-eastern Madagascar seem to have particularly small ranges (Wollenberg *et al.* 2008; Vences *et al.* 2010), whereas some other species in eastern Madagascar, such as the poison frog *Mantella baroni*, share the same haplotypes in populations spread across hundreds of kilometers (Rabemananjara *et al.* 2007a). Similarly, several frog species in arid western Madagascar are widespread, such as *Boophis doulioti*, *Blommersia* sp. aff. *wittei*, *Mantidactylus* sp. aff. *ulcerosus* “Isalo”, *Heterixalus luteostriatus*, and others, most of which reproduce in lentic water bodies. These species probably are able to survive in and maintain gene flow across largely

unforested, human-modified areas. On the contrary, a higher degree of microendemism may apply to species reproducing in permanent or temporary streams, or independently from open water bodies. For instance, the three cophyline microhylids known from Bemaraha (*Plethodontohyla fonetana*, *Stumpffia* sp. aff. *helenae*, and *Rhombophryne* sp.; see Glaw & Vences 2007; Andreone & Randrianirina 2008) have so far not been found elsewhere in Madagascar and probably are endemic to this limestone massif, similar to the stream-breeding *Boophis tampoka*. Also, none of the microhylids found in the transitional forest of Berara in the northwest (Andreone *et al.* 2001) has so far been found in either Bemaraha or Isalo (Glaw & Vences 2007). Herpetofaunistic relationships between Isalo and Bemaraha are indicated by the presence of the mantellid frog candidate species *Blommersia* sp. aff. *wittei* "Isalo" and *Mantidactylus* sp. aff. *ulcerosus* "Isalo" in both the Isalo and Bemaraha regions (candidate species names from Glaw & Vences 2007), whereas both species are unknown from the Berara region (Andreone *et al.* 2001; Glaw & Vences 2007). These species do not appear to show high genetic differentiation between Bemaraha and Isalo according to our preliminary unpublished analyses.

Rabemananjara *et al.* (2007b) showed that the *Mantella betsileo* group has genetically differentiated populations along a north-south axis in western Madagascar, with specimens from Isalo (*M. betsileo* and *M. expectata*) and Berara (*M. ebenau*) belonging to different species, and specimens from Bemaraha being different in having a haplotype related to *Mantella expectata*. Similarly, in the genus *Gephyromantis*, Isalo is populated by *Gephyromantis azzurrae* and *G. corvus* (Mercurio & Andreone 2007), Berara by *G. pseudoasper* (Andreone *et al.* 2001), and Bemaraha harbours a yet undescribed but confirmed candidate species that is phylogenetically more closely related to the Isalo species than to *G. pseudoasper* (Vieites *et al.* 2009). Our analysis indicates that the *Boophis albilabris* group in western Madagascar conforms to the pattern shown by these two groups of species: the form occurring in Berara is the genetically most divergent one and differs from *B. occidentalis* from the type locality (the Isalo Massif) in morphology and advertisement calls, justifying its inclusion in a separate species, *Boophis tsilomaro*. The population from the Tsingy de Bemaraha appears to be genetically closer to Isalo, albeit showing a significant genetic and probably also morphological differentiation. Future studies need to focus on obtaining a larger series of adult specimens from Bemaraha and to record the advertisement calls from this population to understand if it is a divergent population (deep conspecific lineage; Vieites *et al.* 2009) of *B. occidentalis* as we assume here, or represents a third, yet undescribed, western species of the *B. albilabris* group.

Our molecular tree (figure 1), which is an extension of a phylogeny provided by Glaw *et al.* (2010), furthermore includes sequences of *Boophis praedictus* from Vevembe and from Makira forest, and from a small-sized specimen with an undulating white stripe along the upper lip. This latter specimen, from Masoala, has been discussed as candidate species *Boophis* sp. aff. *albilabris* "reticulated lip" in Glaw & Vences (2007) and as *Boophis* sp. 6 in Vieites *et al.* (2009). Because the sequence from this specimen was identical to that of an adult female of *B. praedictus* from Makira forest (geographically close to Masoala), we here conclude that very probably the specimens with undulating white lip are subadults of *B. praedictus* rather than a separate species.

*Boophis occidentalis* has been classified as Near Threatened according to IUCN criteria (Andreone *et al.* 2005, 2008). Despite its apparently large extent of occurrence, it was concluded that its area of occupancy is relatively restricted, and habitat degradation in the dry and transitional forests of western and north western Madagascar is ongoing at a high pace. Because we here consider the Bemaraha population as a deep conspecific lineage, the general status of *B. occidentalis* should remain unchanged despite the exclusion of the Berara population from this species. On the contrary, the new species *B. tsilomaro* is so far only known from the transitional forest of Berara which is under high human pressure. Another apparent Berara endemic frog (*Cophyla berara*) has been recently (IUCN Redlist 2009) classified as "Critically Endangered" because its extent of occurrence is less than 100 km<sup>2</sup>, all individuals are in a single location, and there is continuing decline in the extent and quality of its habitat. *Boophis tsilomaro* is a large and conspicuous species that so far has not been found elsewhere; under the current state of knowledge, its situation is therefore identical with that of *Cophyla berara*. We therefore propose to classify *Boophis tsilomaro* as "Critically Endangered".

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